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PRINCIPAL INVESTIGATOR: Masaaki Hamaguchi, M.D., Ph.D.

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

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Masaaki Hamaguchi, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

E-Mail: hamaguch@cshl.org

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U.S. Army Medical Research and Materiel Command
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13. ABSTRACT (Maximum 200 Words)

Breast cancer arises through the accumulation of genetic alterations that affect two classes of genes, ongogenes and tumor suppressor genes. These genes must be identified for several reasons. Characterization of the genes that drive carcinogenesis will facilitate a better understanding of cancer development. As part of this big picture, we have studied tumor suppressor genes involved in breast cancer. A tumor suppressor candidate, DBC2 was analyzed. DBC2 is composed of a RAS domain, protein-protein interacting domains, and DNA binding domain. DBC2 expression is extinguished by hypermethylation in more than half of breast tumors we tested. When DBC2 expression was induced in tumor cells, the cell growth was expressed. Additionally, mutants we discovered in breast cancer specimens were found to lack the tumor suppressor function. We are studying DBC2's physiological roles by expression microarray analysis, protein-protein interaction studies, and functional analysis. We have identified several genes upregulated by DBC2 and potential candidates for interaction. We have also established techniques to control DBC2 expression, both induction and suppression, which will facilitate further analysis of DBC2.

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INTRODUCTION

In the course of carcinogenesis, cells acquire a number of genetic changes that are recorded in their genomes, most of which have no effect on cellular function. There are, however, critical genetic alterations that affect two classes of genes: oncogenes that are activated or altered in function; and tumor suppressor genes that are down-regulated or abolished in cancer cells (1). These alterations include amplifications, deletions, insertions, translocations, single nucleotide substitutions, hypermethylation, and chromosomal abnormalities. We have discovered a new tumor suppressor gene DBC2 within the homozygously deleted region in breast cancer. Further investigation of DBC2 may lead to a better understanding of tumorigenesis and more effective diagnosis and treatment of breast cancer.

BODY

Approved Statement of Work

Task 1. To narrow down the commonly deleted region in breast tumors (months 1-6)

    ◊ generate STS markers in the deletion epicenter (months 1-2)
    ◊ perform deletion mapping of tumor pairs (months 3-6)
    ◊ identify the breakpoint in each tumor cell (months 5-6)

Task 2. To construct a transcript map of the commonly deleted region (months 1-18)

    ◊ identify transcripts from the region (months 1-9)
    ◊ isolate full-length cDNA (months 7-18)

Task 3. To characterize the genes affected by the deletion (months 19-30)

    ◊ screen expression pattern of the genes (months 19-24)
    ◊ search for mutations of the genes in breast tumors (months 25-30)

Task 4. To study the biology of candidate genes for tumor suppressor (months 30-48)

    ◊ make antibody of the gene product (30-36 months)
    ◊ examine the changes of phenotype by the induction of the genes (30-36 months)
    ◊ RNA profiling of the cells before and after the induction (30-36 months)
    ◊ determine localization of the gene product (37-39 months)
    ◊ Examine biological function of the gene product (30-48 months)

Task 1

Task 1 was completed. The following is a brief summary of the outcome.

Characterization of the deletion locus

We have discovered homozygously deleted regions in breast cancer using RDA (Representational Difference Analysis). Among them, a deletion at human chromosome 8p21 was investigated further. Sixty STS's mapped to the locus were used to construct physical and deltion maps. The commonly deleted region was narrowed down to approximately 1.2 Mb.

LOH Analysis

Loss of heterozygosity (LOH) study was also performed. Of tumors from the 73 patients informative for D8S1734, 20 (27 percent) had lost one allele at the marker.

Task 2

Task 2 was completed. The following is a brief summary of the outcome.

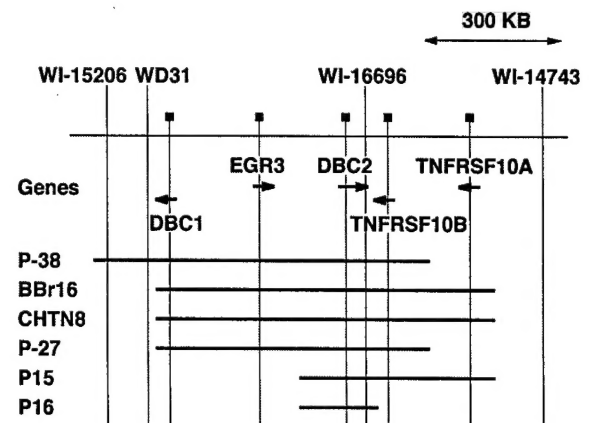*Isolation of Transcripts from 8p21 Locus*

Seven genes were isolated from the commonly deleted region (Figure 1). They were TNFRSF10B, TNFRSF10C, EGR3, BIN3, PHYHIP, DBC1 and DBC2 (accession number AY009093). The full length cDNA of DBC1 and DBC2 were registered to GenBank (Accession numbers AF293335 and AY009093, respectively) Full-length cDNA of these genes were cloned. We further analyzed TNFRSF10B, DBC1 and DBC2 since the other genes were less likely to be a tumor suppressor gene. PHYHIP eventually turned out to be out side of the deleted region. EGR3-deficient mice are not susceptible to tumorigenesis. TNFRSF10C is a decoy receptor and its disruption may be beneficial for tumor cells.

Figure 1. Deletion map of 8p21. The vertical lines indicate the localization of STSs and genes used for deletion mapping, all of which were tested against 200 tumor samples. Genes are represented by a horizontal line with an arrowhead. The STSs are listed above the line and the lines with a square indicate probes derived from the genes. The arrow with double heads indicates approximately 300 kb. Horizontal lines in the lower half represent deletions found in the tumor samples (named to the left). The DBC2 probe and WI-16696 demonstrated the highest deletion frequency.

Task 3

Task 3 was completed. The following is a brief summary of the outcome.

Mutation Analysis

We did not detect significant mutations in DBC1 or TNFRSF10B by DHPLC (denatured high performance Liquid Chromatography) and SSCP (single strand conformation polymorphisms) analyses. In contrast, we discovered two somatic mutations in breast cancer specimens and two additional missense mutations in lung and breast cancer cell lines. In total, from cell lines and tumors, four missense mutations in the coding region of DBC2 were found.

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Computational analysis of DBC2

To the best of our knowledge, DBC2 is a previously unreported gene and a member of a RAS-BTB gene family.

Expression Studies

Expression studies of DBC2, DBC1, and TNFRSF10B were performed by RT-PCR and RNA blotting. We find that all three genes are expressed ubiquitously in all normal sources. However, DBC2 expression is extinguished in about half of breast and lung cancer specimens, but not in colon or other cancers we examined. In contrast, neither TNFRSF10B nor DBC1 expression is substantially extinguished in cancers from any source.

Task 4

Subcellular localization of DBC2

This was completed in previous years. Using DBC2-green fluorescent protein (GFP), we demonstrated that DBC2 was localized in the nucleus (Figure 2).

Inducible Gene Expression System

To study effects of DBC2 induction in breast cancer cell lines lacking the DBC2 transcript, we utilized an inducible gene expression system (2). Four retroviral vectors (a control, a gene transfer, and two receptor vectors) are used in the retrovirus-mediated ecdysone inducible gene expression system. When the host cell line is treated with an ecdysone analog, muristerone A, the receptors form complexes and activate ecdysone responsive elements (E/Gre). The activated E/Gre promotes transcription of a gene under a minimal heat shock promoter. To establish stable host cell lines, several cell lines including 293, HeLa and T-47D were transfected with retroviral vectors with ecdysone receptors. We then transformed the host cells with E/Gre-GFP and analyzed them by a fluorescent activated cell sorter (FACS) without induction in order to eliminate cells with leaky expression of the transgene. The remaining cells were treated with muristerone A and then analyzed by FACS. The GFP-positive cells were collected and verified by microscopic observation. The selected host cells had no detectable expression of GFP before induction but, after induction, had substantial expression of GFP. DBC2 expression was successfully induced by this system and the consequences were investigated (Figure 3A).
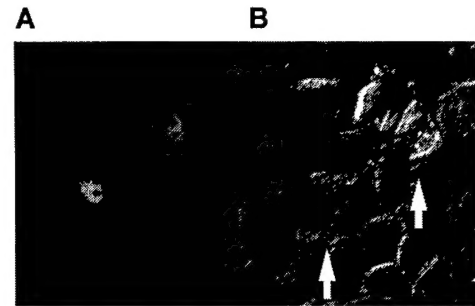
Figure 2. Subcellular localization of DBC2-GFP fusion proteins. *A.* DBC2-GFP (green fluorescent protein) fusion proteins were transiently expressed by a CMV promoter. GFP signals were observed mainly in nuclei. *B.* Phase contrast of the same field is shown. Arrows indicate cells that appear in A.
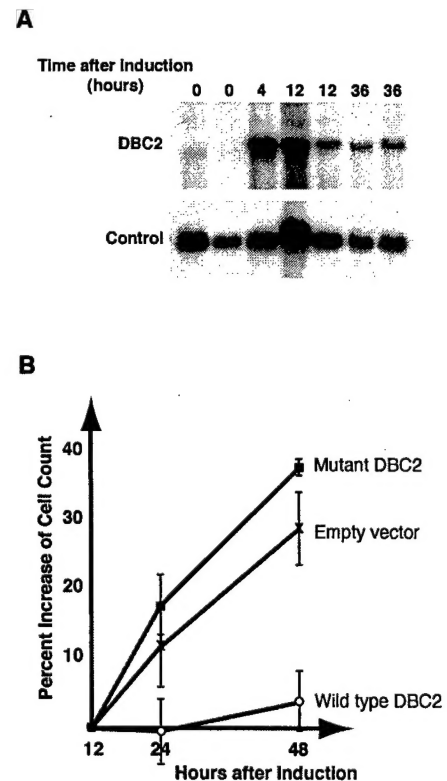
Figure 3. *A.* **RNA blot analysis.** DBC2 expression in T-47D cells was detected only after induction. *B.* **Growth curves of a breast cancer cell line, T-47D.** The ordinate is percent increase of cell count. The abscissa is hours after induction. After induction of empty vector (x) and a DBC2 mutant (squares), the tumor cells kept growing. In contrast, the tumor cells stopped growing after induction of wild type DBC2 (circles).

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DBC2 Induction in Breast Tumors

We demonstrated that induction of DBC2 had no effect on growth of HeLa cells, which expressed endogenous DBC2. In contrast, growth of T-47D cells was greatly hindered by DBC2 induction (Figure 3B). The next question was if mutated DBC2 could also suppress tumor growth. We generated mutant DBC2 fragments containing the same missense mutations we discovered in breast cancer specimens. Induction of the mutants did not show growth inhibition (Figure 3B). The cancer cells with mutated allele have LOH of DBC2 locus, indicating absence of wild type DBC2. These finding indicate that DBC2 is the target of the genetic alterations at 8p22 (3).

Biological function of DBC2

To analyze functional domains of DBC2, we performed a framework. Since the BTB domain is predicted to serve as a protein-protein interacting domain, isolation of proteins that interact with DBC2 is important. We utilized yeast two hybrid systems and immunoprecipitation to achieve this goal. We have four candidate interacting genes. We are in the process of confirmation of these interaction.

The RAS domain shows significant homology to know G-proteins. We first studied DBC2's GTP-binding ability and GTPase activity. DBC2 was found to bind and catalyze GTP. Since some mutations are found to alter the function of RAS proteins, we made equivalent mutations in the RAS domain by site-directed mutagenesis. We are planning to perform functional studies of these mutants.

RNA profiling

We are currently analyzing expression patterns of T-47D using expression microarrays containing 46,000 probes. The acquired data are statistically processed with a computer program (S-Plus) and alterations of gene expression level are examined. RNA profiles of T-47D cells with inducible DBC2 are compared with those with an empty vector. We have identified a number of genes that were up-regulated (Figure 4).

Generation of anti-DBC2 antibodies

Generation of antibodies is an essential step to study new protein. DBC2 protein is expressed in *E. coli* for protein purification. We had difficulties with purification of DBC2 protein mainly because it was not easy to solubilize DBC2 protein. However, we now have partial DBC2 proteins as well as whole protein that can be used as immunogen. We will generate both polyclonal and monoclonal antibodies.
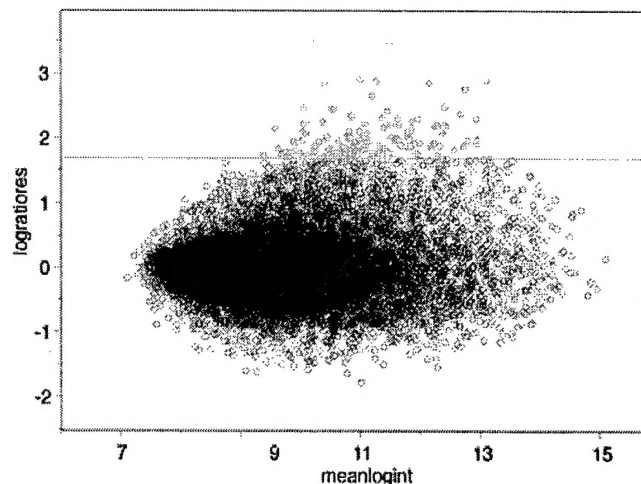
Figure 4. Comparison of expression patterns of the cells with and without DBC2 induction. The ordinate is "logratio of signal intensity" that represents changes of gene expression level. The base of log is two. The abscissa is "log of mean intensity", which represents abundance of the gene in the cell. Red circles represent genes whose expression is increased by more than three folds after DBC2 induction.

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Future experiments

1. We will generate anti-DBC2 antibodies.
   Generation of antibodies will eliminate the necessity to use epitope tags and possibly reduce the risk of using functionally fusion proteins.

2. We will study protein-protein interaction of DBC2.
   The candidate proteins for interaction will be thoroughly studied. Once the interaction is confirmed, it will provide a clue to clarify biological pathways DBC2 is involved.

3. We will establish DBC2-deficient animal models.
   Establishment of animal models is a critical step to study DBC2. Since DBC2 has no counterparts in *C. Elegans* or *D. melanogaster*, a mouse model is a reasonable choice. Mouse cDNA and genomic sequence were cloned and sequenced. We have enough information to generate gene-targeting clones. We will generate DBC2 knockout mice.

4. We will examine DBC2's DNA binding capabilities.
   Since 3' end of DBC2 demonstrates a weak homology to Zinc finger gene and DBC2 localizes in the nucleus, it is logical to test its DBA-binding capability.

Key research Accomplishments

- Physical and deletion mapping of 8p22
- Isolation of six genes that are affected by deletions in breast cancer cells
- Discovery of a new tumor suppressor gene DBC2
- Genetic analysis of DBC2
- Functional analysis of DBC2
- Establishing technique to manipulate DBC2 expression
- Identification of the mouse counterpart Dbc2
- Isolation of genomic sequence of human DBC2 and mouse Dbc2.

Reportable Outcomes

Publication
- Hamaguchi M., Meth J. L., von Klitzing C., Wei W., Esposito D., Rodgers L., Walsh T., Welcsh P., King M. C. and Wigler M. H., "DBC2, a candidate for a tumor suppressor gene involved in breast cancer." *Proc Acad Natl Sci USA* **99:** 13647-13652, 2002.

Presentations
- Era of Hope 2002 (October, 2002)
  "Cloning of Tumor Suppressor Genes for Breast Cancer"

- The 62nd Annual Meeting of Japanese Cancer Society (September 2003)
  "Expression of Tumor Suppressor Gene DBC2 in Breast Cancer Cells Causes Growth Arrest"

Database
- DBC1: Accession Numbers, AF293335, NM02174, AAG02472.
- DBC2: Accession Numbers, AY009093, AF315385, AAK07562
- Full length cDNA of mouse DBC2: Accession Number, AF420001
- Genomic sequence of mouse DBC2: Accession Number, AF420002

Development of cell lines
- DBC2 inducible cell lines derived from T-47D cells.

Funding applied for based on work supported by this award
- NIH (1R01CA100006-01A1): Funded
  "Genetic Analysis of DBC2"
- CDMRP (BC043036): Pending
  "Studies of Pathophysiological Functions of a Tumor Suppressor Gene DBC2"

Conclusions

All tasks in the "Approved Statement of Work" are accomplished except generation of antibodies that we are currently preparing to perform.

We have discovered homozygous deletions at 8p22 in breast cancer. Deletion mapping revealed that the deletion epicenter lies in the region of 200 kb at 8p22. Seven genes are shown to be involved in more than one deletion. We have isolated full-length cDNA of these genes including two novel genes, which have been registered in public databases. Genetic and functional analyses of these genes indicated that DBC2 was the target of the 8p22 genetic alterations. Namely, DBC2 expression is extinguished in more than half of breast tumors we tested. Methylation analysis of tumor cells revealed hypermethylation of the CpG islands in the DBC2 promoter region. Additionally, we have discovered in breast cancer specimens somatic mutations of DBC2 that are accompanied by LOH. When DBC2 expression was induced in tumor cells, the cell growth was hindered whereas mutated DBC2 failed to suppress growth of the tumor cells.

DBC2 is a structurally new gene and its function remains to be studied. DBC2 is composed of a RAS domain, protein-protein interacting domains, and DNA binding domain. We hypothesize that DBC2 is a G protein-coupled transcriptional regulator. To test this hypothesis, we are conducting functional analysis of DBC2, including isolation of interacting proteins, GTP-binding/GTPase-activity assays, and RNA profiling of DBC2 induced/non-induced cells. We will continue studying patho-physiological functions of DBC2 and establish animal models in order to clarify the mechanism of breast cancer development.

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2. Stolarov, J., Chang, K., Reiner, A., Rodgers, L., Hannon, G. J., Wigler, M. H., and Mittal, V., Design of a retroviral-mediated ecdysone-inducible system and its application to the expression profiling of the PTEN tumor suppressor. *Proc Natl Acad Sci U S A* **98**:(23)13043-8 (2001).

3. Hamaguchi, M., Meth, J. L., Von Klitzing, C., Wei, W., Esposito, D., Rodgers, L., Walsh, T., Welcsh, P., King, M. C., and Wigler, M. H., DBC2, a candidate for a tumor suppressor gene involved in breast cancer. *Proc Natl Acad Sci U S A* **99**:(21)13647-13652 (2002).

DBC2, a candidate for a tumor suppressor gene involved in breast cancer

Masaaki Hamaguchi*†, Jennifer L. Meth*, Christine von Klitzing*, Wen Wei*, Diane Esposito*, Linda Rodgers*, Tom Walsh‡, Piri Welcsh‡, Mary-Claire King‡, and Michael H. Wigler*

*Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724; and ‡Departments of Medicine and Genome Sciences, University of Washington, Seattle, WA 98195-7720

A previously uncharacterized gene, DBC2 (deleted in breast cancer), was cloned from a homozygously deleted region at human chromosome 8p21. DBC2 contains a highly conserved RAS domain and two putative protein interacting domains. Our analyses indicate that DBC2 is the best candidate tumor suppressor gene from this region. It lies within the epicenter of the deletions and is homozygously deleted in 3.5% (7/200) of breast tumors. Mutation analysis of DBC2 led to discovery of two instances of somatic missense mutations in breast tumor specimens, whereas no missense mutations were found in other candidates from the region. Unlike other genes in the region, expression of DBC2 is often extinguished in breast cancer cells or tissues. Moreover, our functional analysis revealed that DBC2 expression in breast cancer cells lacking DBC2 transcripts causes growth inhibition. By contrast, expression of a somatic mutant discovered in a breast cancer specimen does not suppress the growth of breast cancer cells.

I n the course of carcinogenesis, cancer cells acquire a number of genetic changes, many of which may have no effect on cellular function. There are, however, critical genetic alterations that affect two classes of genes: oncogenes that are activated or altered in function and tumor suppressor genes that are down-regulated or ablated in cancer cells. Discovery and analysis of these genetic changes have contributed to a better understanding of the molecular basis of cancer development. Studies on BRCA1 and -2 tumor suppressor genes have provided useful information for understanding familial breast cancer (1). Other tumor suppressor genes are involved in sporadic breast cancer, including PTEN and p53 (2, 3). However, it is likely that many, if not most, tumor suppressor genes responsible for sporadic breast cancer remain to be discovered. Representational difference analysis (RDA) is one tool that has been shown to be effective in the hunt for tumor suppressor genes (3, 4). We have analyzed a few breast cancer biopsies by RDA and isolated a number of deletion probes. An RDA probe detecting homozygous deletion was mapped to human chromosome 8p21, and we find this region to be deleted in other breast cancer specimens as well. We have mapped this region, cloned new genes residing therein, and have found somatic mutations in cancer cells for one of the genes from this locus.

Materials and Methods

Materials. Breast tumor specimens were procured from a variety of sources. Aneuploid and diploid nuclei from breast tumor specimens were prepared by fluorescence-activated sorting by using an Elite EPS (Beckman Coulter). DNA from the sorted nuclei was immortalized by making DpnII and BfaI representations, by using methods described elsewhere (5). Cell lines were purchased from American Type Culture Collection or are from our permanent collection inherited from Jorgen Fogh (Memorial Sloan–Kettering Cancer Center). Chemicals and buffers were obtained from Sigma unless otherwise noted. Enzymes were obtained from New England Biolabs except TaqDNA Polymerase (AmpliTaq), from Perkin–Elmer. All enzymatic reactions were performed with buffers recommended by the supplier.

Deletion Analysis. Deletion mapping was performed by quantitative PCR (Q-PCR) with the ABI Prism 7700 Sequence Detector (6). The Q-PCR was carried out in three stages: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. DNA markers from 8p21 and other chromosomes were used for Q-PCR. DNA markers were derived from the original RDA probe WD31; from sequence tag sites WI-14743, WI-15206, and WI-16696; from genes we discovered [deleted in breast cancer 1 (DBC1) (GenBank accession no. AF293335) and deleted in breast cancer 2 (DBC2) (accession no. AY009093)]; and from known genes from this region, including EGR3 (accession no. NM004430), tumor necrosis factor receptor super family 10B (TNFRSF10A) (accession no. AF014794), TNFRSF10B (accession no. AC007868), and KIAA0273 (accession no. NM014759). Additionally, DNA probe ER48 was a subcloned fragment from a genomic clone from human chromosome 3p14, and WB23 was a cloned fragment from human chromosome 21p.

The protocols for RDA are described elsewhere (4). All oligonucleotides were synthesized by Operon Technologies (Alameda, CA), except that Taqman probes for Q-PCR were synthesized by Perkin–Elmer with 6-carboxyfluorescein at the 5′ end and 6-carboxy-tetramethyl-rhodamine at the 3′ end.

Gene Cloning. Gene discovery methods included exon trapping (7), rapid isolation of cDNA by hybridization (8), cDNA library screening, and sequence determination of genomic clones followed by database searches. RNA ligase-mediated amplification of cDNA ends (RLM-RACE) was performed by using the First Choice RLM-RACE kit (Ambion, Austin, TX) (9). Computational analyses of the fragments were performed by BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST), GCG Wisconsin Package, and a PFAM HMM search (10, 11).

Mutation Analysis. Mutational analyses were performed by a protein truncation test (PTT), denatured HPLC (DHPLC), and single-strand conformation polymorphism (SSCP). For PTT, the entire ORF of TNFRSF10B was amplified with primers, TAATACGACTCACTATAGGGAGCCACCATGGAACAA-CGGGGACAG and AATTGTGGCACTTTCCTACTGACT. The PTT procedure is described elsewhere (12). WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, NE) was used for DHPLC (13). The target fragments were amplified by PCR with the GeneAmp PCR System 9600 (Perkin–Elmer). Control fragments were generated by PCR from normal human

DNA. The samples and control fragments were mixed, denatured, reannealed to generate heteroduplexes, and then analyzed by DHPLC. The samples with different peak patterns were sequenced. Protocols for SSCP are described elsewhere (14). The primer sequences for Q-PCR, DHPLC, and SSCP used in this study will be provided on request.

Expression Analysis. Expression analyses were mainly done by RT-PCR. Cytoplasmic RNA was extracted from cultured cells, and total RNA was extracted from tissue samples with guanidinium thiocyanate followed by centrifugation in cesium chloride solutions. The protocols are described elsewhere (15). RNA of normal tissues was either purchased from BD Biosciences (Palo Alto, CA) or extracted from surgical specimens. Avian myeloblastosis virus reverse transcriptase purchased from Boehringer Mannheim was used to synthesize cDNA with oligo-dT primers. PCR was then performed with AmpliTaq. The 50-μl PCR reaction mixture contains 1 μl of the cDNA, 200 μM of each dNTP, 1 μM of each primer, 2 units of AmpliTaq, and 1× AmpliTaq buffer. After initial denaturation at 94°C for 10 min, 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 3 min were performed. The primer sequences used in this analysis were: AACATGGAGGCCCTGTGCCAGCAGAA and ACTCGCAGCTCCTGCATCTCT (DBC1); ACCATGTGG-TACCCAGAAATC and TTAGACAACAGCAGCAGGGT-CAGA (DBC2); AGTTTGACCAGAGATGC and TGAC-CAAGGCTGAATAAATCCC (TNFRSF10A); CTTGATTGT-GGCTGTGTTTGTT and GCCACCTTTATCTCATTGTCCA (TNFR10B); AGCATGGAGCTGCTGTCCAC and TTAGCT-CCCTGAACCTGGGCTACC (KIAA0273); and CCATGGAG-GAGCCGCAGTCAGA and TTAGTCTGAGTCAGGCCCT-TCTGT (p53).

Mutagenesis. Mutation was introduced by using the Exsite Mutagenesis kit (Stratagene). Sequences of primers used for mutagenesis were ATGAACCTGAGTGAGGGGGAGCT and CAGCTCCCCCTCACTCAGGTT. The generated mutation was confirmed by sequencing of both strands.

Gene Expression. HeLa and T-47D cells were grown in DMEM supplemented with 15% FCS. For constitutive gene expression, a mammalian expression vector pcDNA3.1/His (Invitrogen) was used. For inducible gene expression, an ecdysone gene expression system was used. Stable host cell lines carrying ecdysone receptors were established by using retroviral vectors (16). The genes of interest were cloned into pIND (Invitrogen) with an Xpress tag at the 5′ end and transfected into the host cell lines. All constructs were verified by sequencing. The cells were transformed with 15 μg of plasmid by electroporation (field strength = 0.25 kV/cm, capacitor = 950 μF) using Gene Pulser II (Bio-Rad) and selected with G418 (400 μg/ml) and Zeocin (Invitrogen) (150 μg/ml). Muristerone A (Invitrogen) (5 μM) was administered to induce the genes in pIND. Cell growth was monitored by using the CyQuant Cell Proliferation Assay kit (Molecular Probes).

Sequencing. DNA sequencing was performed with the ABI Thermal Cycler Sequencing kit and ABI Prism 377 DNA Sequencer (Perkin–Elmer). Both strands were sequenced unless otherwise specified.

Results

Homozygous Deletion. RDA of breast cancer samples generated a number of deletion probes, including WD31 from a tumor called BBr67. Homozygous deletion (HD) of WD31 in BBr67 was confirmed both by Southern blotting of the DNA representations made from the aneuploid nuclei of BBr67 and by the semiquantitative PCR analysis of genomic DNA made from
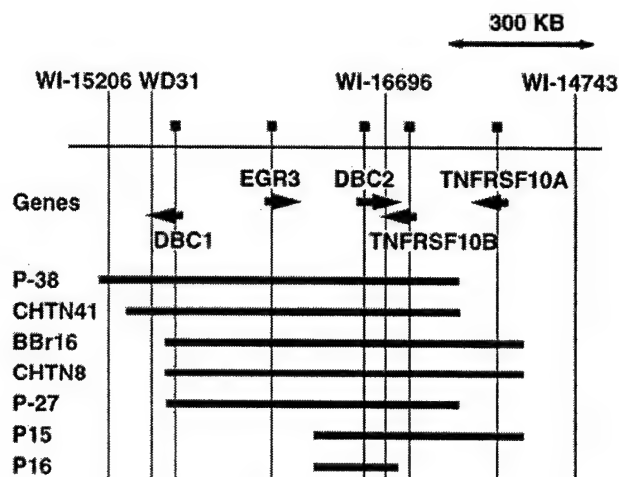
Fig. 1. Deletion map of 8p21. The vertical lines indicate the localization of sequence tag sites and genes used for deletion mapping, all of which were tested against 200 tumor samples. Genes are represented by a horizontal line with an arrowhead. The STSs are listed above the line, and the lines with a square indicate probes derived from the genes. The arrow with double heads indicates approximately 300 kb. Horizontal lines in the lower half represent deletions found in the tumor samples (named, *Left*). The *DBC2* probe and WI-16696 demonstrated the highest deletion frequency. Both *DBC2* and *TNFRSF10B* genes are overlapping with different orientations and deleted in all tumor specimens.

these nuclei. WD31 was mapped to 8p21 by radiation hybrid panels and genomic library screening, and the locus was scrutinized further. We constructed a contig of genomic clones and applied positional cloning methods, described in *Materials and Methods*. We discovered two previously uncharacterized genes and identified four known genes extending outward from WD31: *DBC1*, *DBC2*, *TNFRSF10A*, *TNFRSF10B*, *EGR3*, and *KIAA0273*. These genes were mapped by us, and our mapping order was confirmed by comparison to the human genome sequence (www.ncbi.nlm.nih.gov/) (Fig. 1).

Deletion Analysis. These genes, as well as nearby sequence tag sites, were then used for deletion analysis. Aneuploid and diploid nuclei from breast cancer were sorted and analyzed to determine deletion frequency and the deletion epicenter. Although aneuploid nuclei will be enriched for tumor cells, and diploid nuclei will be enriched for normal stroma, each fraction will be contaminated to varying extents with each other. Real-time PCR or Q-PCR was used to analyze genetic loss for markers in the region. Even in the presence of low levels of contamination, we can detect a homozygous deletion as a cycle difference between aneuploid and diploid samples (Fig. 2). We used multiple control markers for normalization, from chromosome 8 and other chromosomes. For example, we used ER48 and WB23 (from other chromosomes) to standardize the DNA amount and markers on the short arm of chromosome 8 to control for chromosome 8 copy number. In all cases tested, Q-PCR results were confirmed by Southern blotting of representations made from diploid and aneuploid nuclei (Fig. 2). The deletion mapping using 200 breast tumors revealed that the deletion frequency was 3.5%, and the deletion epicenter lay around a DNA marker WI-16696 that was surrounded by two genes. WI-16696 is contained in the intron 7 of *DBC2* and located within 1 kb of the 3′ end of *TNFRSF10B*. On the basis of the transcription and deletion mapping (see Fig. 1), these two genes resided at the deletion epicenter, and hence we studied them further.

A

B

	1	2	3	4

WD31

ER48
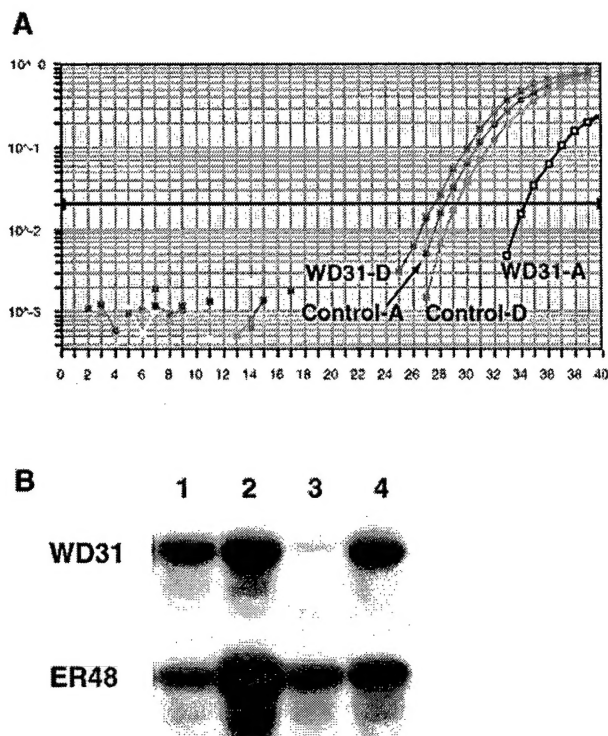
Fig. 2. Deletion analysis of tumor samples. (A) Real-time PCR analysis of a breast tumor sample CHTN41 is demonstrated. The abscissa is cycle number of PCR and the ordinate is quantity of PCR products. The control primer set in this figure is WB23 from chromosome 22. "D" and "A" represent DNA from diploid and aneuploid fractions, respectively. The amplification curve from aneuloid DNA with WD31 primer set has a several cycle difference from the others, indicating deletion of WD31 in this tumor. (B) Southern blot analysis of DpnII representation with WD31 probe is shown. Each lane contains 5 μg of DpnII restriction fragments. Lanes 1 and 2 are DNA from a tumor and a matched normal (CHTN40), respectively, showing no deletion. Lanes 3 and 4 contain DNA from a tumor and a matched normal pair (CHTN41), showing deletion. ER48 is a control probe derived from chromosome 3. Lane 3 has a very faint band for DBC2, whereas the control probe exhibits the same intensity for tumor and normal samples, representing homozygous deletion. Contamination of normal stromal cells is considered to contribute the faint band.

Mutation Analysis. We began our study with *TNFRSF10B*, which encodes the Trail2 receptor for TNF, because mutations in the death domain of *TNFRSF10B* in nonsmall cell lung cancer had been previously reported (17). Mutation analysis of the entire ORF of *TNFRSF10B* was performed on 60 cancer cell lines by both protein truncation test and DHPLC followed by sequencing of variants, but no frameshift, missense, or nonsense mutations were found (see Table 1).

The coding region of *DBC2* was also screened for mutations by DHPLC and SSCP, and variants further studied by sequencing, revealing two missense mutations in the BTB/POZ (broad-complex/tramtrack/bric-a-brac/poxvirus/zinc finger) domain, found in lung and breast cancer cell lines. Ninety-five primary tumors and matched normals were then screened by either DHPLC or SSCP for mutations in either of these two genes (see Table 1). Samples showing variants in retention time (DHPLC) or mobility shifts (SSCP) were sequenced, and tumors were compared with matched normals (Fig. 3). No frameshift, missense, or nonsense mutations were found in *TNFRSF10B*. However, two missense mutations within *DBC2* were again discovered within tumors but not in matched normals, indicating that somatic mutations had occurred. One of these mutations, caus-

Table 1. Frequency of codon-altering point mutations found in three genes in the deleted region

| Gene	Materials	Protein truncation test	SSCP	DHPLC
TNFRSF10B	Cell lines	0/60	NA	0/60
	Primary tumor	NA	0/90	NA
DBC2	Cell lines	0/60	2/60	2/65
	Primary tumor	NA	2/65	NA
DBC1	Cell lines	0/60	NA	0/60
	Primary tumor	NA	NA	NA

NA, not applicable.

ing Asp-299→Asn, was in the BTB/POZ domain and was confirmed by an independent PCR amplification and sequencing reaction. This mutation was used for functional studies described below. In total, from cell lines and tumors, four missense mutations in the coding region of *DBC2* were found (see Fig. 4), but no significant mutations were found in *TNFRSF10B*.

Computational Analysis. To the best of our knowledge, *DBC2* is a previously unreported gene. The cDNA for *DBC2* was obtained by cDNA library screening and completeness confirmed by using RNA ligase-mediated amplification of cDNA ends. PFAM analysis of our sequence revealed *DBC2* contained a RAS domain at its 5′ end and two BTB/POZ domains. The RAS domain of *DBC2* has high homology to that of small GTP-binding proteins (G proteins), including the sites for binding to, and hydrolysis of, GTP (Fig. 4). However, *DBC2* is distinct from the typical RAS family members. First, *DBC2* has additional functional domains. Second, *DBC2* lacks the C-terminal lipid-binding sites that anchor the typical family member to membranes. BLAST analysis revealed two human genes with a similar RAS-BTB/POZ structure: *KIAA0740* and *KIAA0878* (accession nos. AB018283 and AB020685, respectively), the first one with high homology, and several genes in other organisms including *Mus musculus* (accession nos. AF420001, AK005770, and AK006650), *Dro-*

D

CTGTTCCTCATGGACCTGAGTGAG

A

CTGTTCCTCATGAACCTGAGTGAG

↑
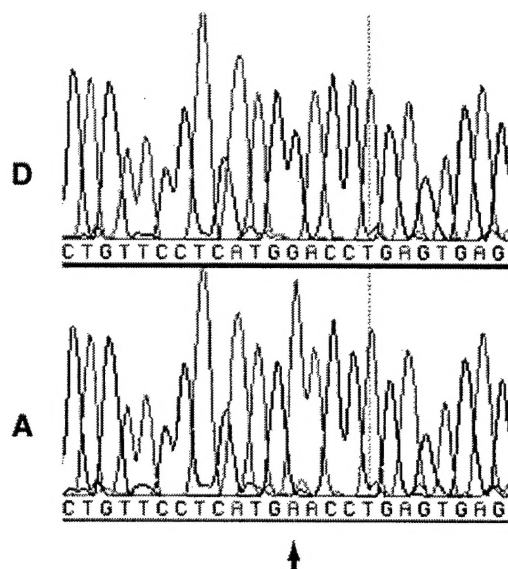
Fig. 3. Electrophoretogram of sequencing analysis. "D" and "A" designate sequencing results of DNA from diploid and aneuploid nuclei of tumor sample CHTN56, respectively. Arrow indicates position of somatic mutation.
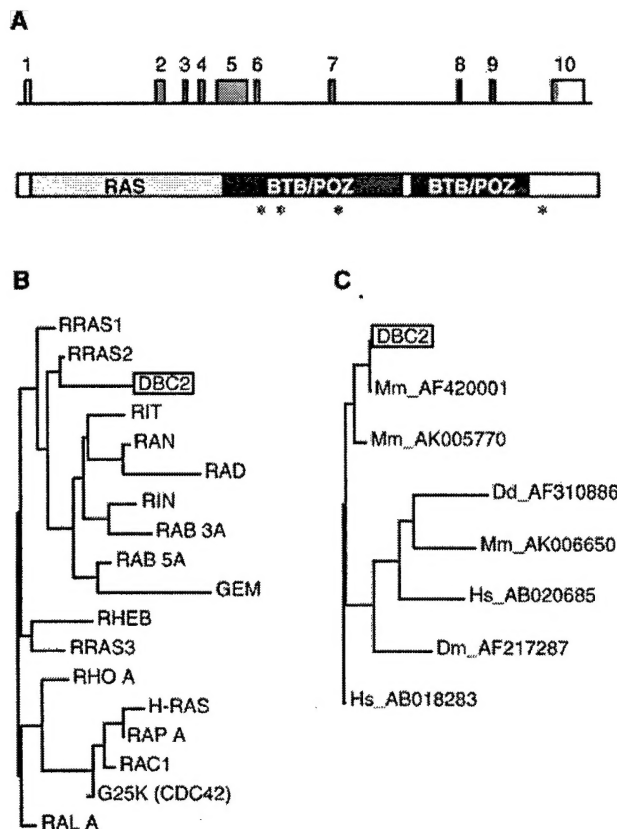
Fig. 4. Structure of *DBC2*. (*A*) The intron–exon structure of *DBC2* (GenBank accession no. AF315385) and predicted functional domains of *DBC2* are shown. The rectangles with a number represent exons. The shaded area indicates coding sequences. Based on deduced amino acid sequence, molecular mass of the predicted *DBC2* product is 83 kDa. The asterisks denote missense mutations we have discovered. There were three in exon 5: T to G (7762), causing Tyr-284→Asp in a lung cancer cell line (SK-Mes-1), G to A (7807), causing Asp-299→Asn in a primary breast tumor (CHTN56), and A to C (8015), causing Asp-368→Ala in a breast cancer cell line (BT483); and one in exon 9: C to A (16389), causing Phe-647→Thr in a primary breast tumor (P-1). (*B*) Phylogenic tree demonstrating overall homology between *DBC2* and human RAS family members is shown. A table of the pairwise distances between the genes was prepared by GCG Wisconsin Package, and a phylogram was then created from the table by using the neighbor-joining method. The length of a horizontal line is proportional to estimated divergence along each branch. Accession numbers of the genes used for this analysis are: *RRAS1* (P10301); *RRAS2* (P17082); *RIT* (AAB42213); *RAN* (P17080); *RAD* (P55042); *RIN* (AAB42214); *RAB3A* (P20336); *GEM* (P55040); *RHEB* (Q15382); *RRAS2* (O14807); *RHO A* (P06749); *H-RAS* (P01112); *RAP A* (P10113); *RAC1* (P15154); *G25K* (P25763); and *RAL A* (P11233). (*C*) RAS-BTB proteins from different organisms are compared. Accession numbers of the genes are listed after the designation of the organism: Hs, *Homo sapiens*; Mm, *M. musculus*; Dm, *D. melanogaster*; and Dd, *Dictyostelium discoideum*.

sophila melanogaster (accession no. AF217287), and *Dictyostelium discoideum* (accession no. AF310886) (Fig. 4).

The BTB/POZ domain functions as a specific protein–protein interaction motif in known proteins (18). It is evolutionarily conserved and found primarily at the N terminus of zinc-finger transcription factors. Many BTB/POZ proteins are transcriptional regulators that mediate gene expression through the control of chromatin conformation; examples are *BACH1* and -*2*, *PATZ*, and *PLZF*, all of which interact with other proteins through their BTB/POZ domains. Unlike these proteins, *DBC2* has two BTB/POZ domains located at the C terminus.
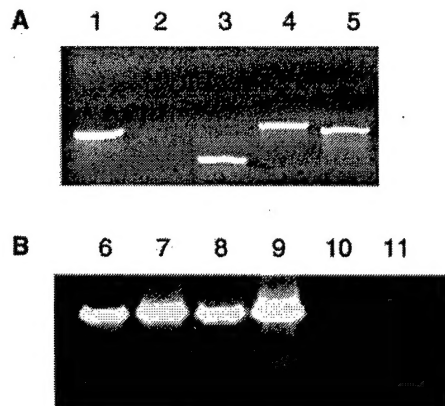
Fig. 5. RT-PCR analysis of tumor specimens. (*A*) RT-PCR products from mRNA of T-47D with five primer sets. Lanes 1–5 contain RT-PCR products with primers of *DBC1*, *DBC2*, *KIAA0273*, *TNFRSF10A*, and *p53*, respectively. Transcripts of all genes but *DBC2* were detected in T-47D. (*B*) RT-PCR products with the *DBC2* primer pair. Lanes 6–11 contain RT-PCR products from NCI-H82, Saos-2, SK-BR-3, SW 982, T-47D, and T84, respectively. *DBC2* expression was observed in NCI-H82, Saos-2, SK-BR-3, and SW 982 but not in T-47D or T84.

Expression Analysis. To gain more support for the hypothesis that loss of *DBC2* may play a role in breast cancer, and possibly other cancers, we examined its expression pattern in a variety of neoplastic and normal cells and tissues. We used RT-PCR to monitor the presence of *DBC2* mRNA, as well as two other genes from its locus, *DBC1* and *TNFRSF10B*. In a subset of cases, we tested our results by RNA blotting, and in all those cases, our RT-PCR results were confirmed. In Fig. 5, we show some representative data, illustrating the lack of expression of *DBC2* in T-47D, a breast cancer cell line. Our data are summarized in Table 2. For neoplastic cells and tissues, we drew on our collection of cultured tumor cell lines and frozen tissue biopsies from various sources, and for normal, we used multiple normal tissue samples, including breast and placenta (*Materials and Methods*). We find that all three genes are expressed ubiquitously in all normal sources. However, *DBC2* expression is extinguished in about half of breast and lung cancer specimens, but not in colon or other cancers we examined. In contrast, neither *TNFRSF10B* nor *DBC1* expression is substantially extinguished in cancers from any source.

Functional Analysis of *DBC2*. To study the function of *DBC2*, we expressed wild-type and mutated *DBC2* in cancer cell lines. We

Table 2. RT-PCR analysis of *DBC2*, *TNFRSF10B*, and *DBC1*

	Tissue of origin	DBC2	TNFRSF10B	DBC1
Neoplasm	Breast	42% (8/19)	89% (17/19)	84% (16/19)
	Lung	50% (7/14)	93% (13/14)	86% (12/14)
	Colon	83% (10/12)	100% (12/12)	92% (11/12)
	Other	91% (10/11)	82% (9/11)	100% (11/11)
Normal	Breast	100% (13/13)	100% (8/8)	100% (8/8)
	Lung	100% (1/1)	100% (1/1)	100% (1/1)
	Placenta	100% (2/2)	100% (2/2)	100% (2/2)
	Brain	100% (3/3)	100% (3/3)	100% (3/3)
	Total	100% (19/19)	100% (14/14)	100% (14/14)

The percentage of the specimens with detectable expression of the indicated gene is listed. The denominator in parentheses indicates the number of specimens tested, and the numerator, the number of positives. Neoplasm specimens are cell lines; normal specimens are tissue.
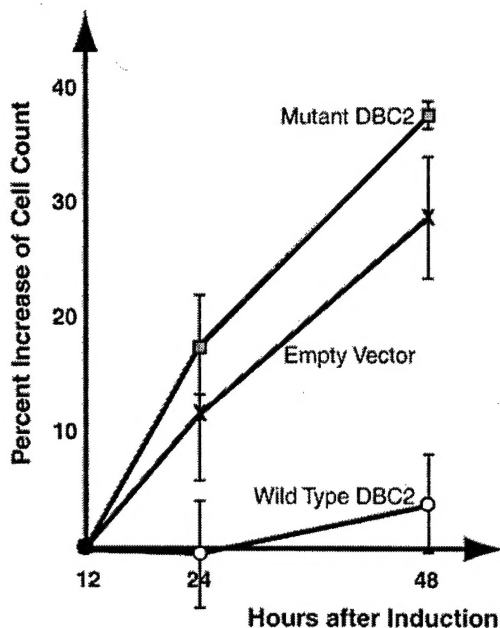
Fig. 6. Effects of gene induction on the growth of T47D. T-47D cells with various constructs were treated with 5 μM Muristerone A. Percent increase of the cell count from 12 h after induction is shown. Expression of wild-type *DBC2* inhibits cell growth of T47D between 12 and 48 h after gene induction, whereas mutant *DBC2* and the empty vector have no influence on T47D proliferation.

Fig. 7. RNA blot analysis of T47D with inducible *DBC2*. T-47D cells with inducible *DBC2* were treated with 5 μM of Muristerone A. *DBC2* expression was examined at various time points after induction. The *DBC2* transcript was detected only after induction. The control is β actin.

generated the G-to-A mutation at nucleotide position 7807 (Asp-299→Asn), which was discovered in breast tumor specimen CHTN56.

First, wild-type *DBC2* and the mutant were cloned into pcDNA3.1/His for constitutive expression. Use of this vector results in expression of an epitope-tagged product. Wild-type and mutant *DBC2* vectors were introduced into HeLa cells that express endogenous *DBC2*. Expression of exogenous genes was verified by Western blot analysis, and neither affected the growth of the HeLa cells. Next we tested T-47D, the breast cancer cell line described above and in Fig. 5. T-47D cells transformed with wild-type *DBC2* were difficult to obtain, and even when transformants were cloned, expression of *DBC2* could not be detected.

We therefore used an ecdysone inducible gene expression system to analyze effects of *DBC2* expression in T-47D, using pIND. T-47D stopped proliferating 12 h after induction of wild-type *DBC2*. Growth suppression lasted for about 36 h. In contrast, induced expression of mutant *DBC2* (Asp-299→Asn) did not suppress growth (Fig. 6). Induced expression was confirmed by RNA blot analysis (see for example Fig. 7).

Discussion

HDs have been a good starting point for the discovery of tumor suppressor genes. Many researchers have tried to detect these HDs by comparing tumors with corresponding normal tissues (19). Arbitrarily primed PCR (APPCR) has been successfully used in some cases, but it can examine only a small portion of genome, because certain combinations of primer will amplify limited numbers of fragments. Furthermore, cloning the differences detected by APPCR is often difficult. Comparative genomic hybridization can screen the entire genome and is a powerful tool for detecting loss of heterozygosity (LOH) and amplification, but its relatively low resolution neither allows HD detection nor provides much help in localizing regions. DNA
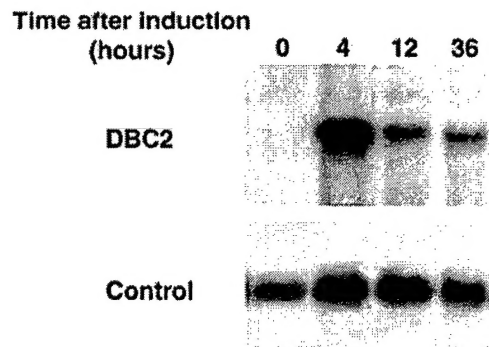
microarray technology may be sufficiently sensitive to detect HD (20), but the true utility of this technique for identifying tumor suppressor genes remains to be established. RDA is currently an effective method to clone HD probes (3, 4), although it too has certain limitations. These limitations include the need for further analysis to distinguish HD from LOH, and that HD probes found by RDA are generally from large deletions. Hence, detailed deletion mapping is required to define the targeted genetic regions. The first problem is not insurmountable. RDA favors detection of HD over LOH, and RDA probes can be further analyzed to distinguish between these events. The second problem is likely to ease as the transcription map of humans becomes better defined.

The initial discovery of an RDA HD probe has resulted in the definition of a region on chromosome 8p that is frequently deleted in breast cancer. The HD region at 8p21, as illustrated here, contains at least six genes. In this report, we have focused on the analysis of two genes, *TNFRSF10B* and *DBC2*, because they reside in the deletion epicenter. There are four other genes that map to this region and are deleted in at least one tumor, but they are less frequently deleted. One of these, *DBC1*, was discovered by us; although it has a long ORF, it shows no homology to any known genes. It is expressed ubiquitously, but we found no point mutations within its coding domains (see Table 1). *KIAA0273*, found on the border of the region, corresponds to a spliced EST with no known function, and we do not find it expressed in normal breast tissue (data not presented). It was not studied further. *EGR3* is a transcription factor implicated in the development of neuromuscular junctions. Knockout mice are not reported to show increased tumorigenicity, and it was not studied further (21). *TNFRSF10A* encodes a decoy receptor for TNF, and we would therefore not suspect it as a tumor suppressor.

In contrast to *EGR3*, *DBC1*, *KIAA0273*, and *TNFRSF10A*, *TNFRSF10B* looked like a reasonable tumor suppressor candidate. *TNFRSF10B* encodes the Trail2 receptor for TNF, and mutations in the death domain of *TNFRSF10B* in nonsmall cell lung cancer had been previously reported. Overexpression of *TNFRSF10B* induces apoptosis of tumor cells in a *p53*-dependent manner (22). However, we did not detect mutations in *TNFRSF10B* in breast cancers, implying either that *TNFRSF10B* is not the tumor suppressor gene involved in breast cancer, or that its inactivation mechanism is not frequently caused by mutations in coding regions. We believe the latter to be unlikely, but *TNFRSF10B* cannot be completely ruled out as a candidate tumor suppressor for breast cancer.

The discovery of missense mutations in the coding region of *DBC2*, residing at the epicenter of deletion, makes *DBC2* the lead candidate for a tumor suppressor gene within the deletion locus at 8p21. Three of four mutations we discovered are located in the BTB/POZ domain and create radical amino acid substitutions likely to change protein function. In fact, functional analysis revealed that *DBC2* suppresses growth of a breast cancer cell line T-47D, but a mutant (Asp-299→Asn) does not. If we sum all deletions and point mutations, we find genetic alteration of *DBC2* in nearly 10% of breast cancer samples. Furthermore, our work indicates that, whereas *DBC2*, *TNFRSF10B*, and *DBC1* are all expressed in normal breast tissue, only expression of *DBC2* is frequently extinguished in breast cancers (11/19).

All these data suggest the involvement of *DBC2* in breast cancer, but its involvement in other cancers is not excluded. In fact, mutation in *DBC2* was found in one lung cancer cell line, and *DBC2* expression was found extinguished in lung cancers.

DBC2 expression is not suppressed in colon cancer or other types of tumors we have tested. These findings imply that *DBC2* may function as a tumor suppressor in a tissue specific manner. A possible explanation is that *DBC2* tumor suppressor function may be circumvented by alternative biological pathways in certain tissues. The existence of altered response to *DBC2* is supported by the observation that nearly half of breast cancer cells express *DBC2*. This hypothesis will also explain resistance of HeLa cells to growth inhibition caused by *DBC2* expression.

The structure of *DBC2* suggests that it encodes a signal transduction protein. It encodes a protein with significant RAS homology and contains known protein–protein interaction domains. Its novel combination of structural domains, lack of a membrane anchor sequence at the C terminus, and divergence from RAS proteins with known functions suggest that its function may be distinct from other members of the superfamily. Curiously, although *DBC2* is an evolutionarily conserved protein, with homologs *M. muculus* and *D. melanogaster,* homologs are not found in all eukaryotes and are conspicuously absent in *Caenorhabditis elegans* (23) and *Saccharomyces cerevisiae* (http://genome-www.stanford.edu/Saccharomyces/). Further work is clearly warranted to illuminate the role of *DBC2* in normal and pathophysiology.

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